REMARKS/ARGUMENTS

Upon entry of this amendment, Claims 25, 30-36, 39-42, 50, 52, 55, and 57-58 are pending. Claims 1-24, 26-29, 37-38, 43-49, 51, 53-54, and 56 including claims subject to restriction and non-elected, have been canceled.

Claim 25 is amended to advance prosecution by eliminating the "epitope" language asserted by the examiner to be new matter. Claim 57 is amended to make it an independent claim by incorporating the features of claim 50 from which it had previously depended. Because dependent Claim 57 has not been rejected on any ground by the current Office Action, amended claim 57 is believed to be in condition for allowance.

Applicants reserve the right to prosecute the non-elected claims and subject matter voluntarily removed from the pending claims in a divisional or continuation application filed during the pendency of the present application.

I. Double Patenting

Claims 30, 31, 50, and 55 are rejected under the judicially created doctrine of obviousness-type double patenting over claims 1 and 2 of US Patent No. 6,610,306.

A terminal disclaimer was filed in this application on September 21, 2006 directed to the '306 patent and already forms part of the record. Therefore, this ground for rejection set out in paragraph 10 of the Office Action is satisfied and may be withdrawn. All pending claims are free of this rejection.

II. 35 USC §112, First Paragraph Rejections

The examiner rejects pending claims 25, 39-42, 44-46, 55-56 and 58 for allegedly:

A. *containing subject matter not described in the specification, i.e., new matter, in the pages of the Office Action spanning 4-7. The examiner states that new matter includes not only the addition of wholly unsupported subject*

matter but also, adding specific percentages or compounds after a broader original disclosure, or even omission of a step from a method; and

B. *containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor, at the time of filing, had possession of the claimed invention, i.e., lacking written description.*

Applicants respectfully request reconsideration and withdrawal of these grounds for rejection in view of the following remarks. Cancellation of Claims 44-46 and 56 moots this rejection as to those claims.

Claim 25 has been amended to eliminate the "epitope" language, and acknowledge that it is diagnostic for either *N. meningitidis* or *N. gonorrhoeae* and thus finds support in the original specification. Amended claim 25 and its dependent claims 39-42 are entitled to the priority date of October 1998, as they are supported by the original application. Claim 25 and claims 39-42 are believed to no longer be subject to the new matter or written description rejections.

With regard to Claim 55 and its dependent Claim 58, Applicants again submit that these claims are fully supported in the specification as written and as interpreted by one of skill in the art. The support for these claims is found within the specification as read by one of skill in the art. Claim 55 does not cover <u>any</u> stretch of 8 amino acids that can be found within SEQ ID NO: 4 and shared with other non-meningococcal or gonococcal microorganisms, as alleged in the Office Action by the examiner. Instead Applicants' Claim 55 requires that (1) the polypeptide is isolated; (2) that the polypeptide comprise an epitope of *at least* 8AA of SEQ ID NO: 4; ***and*** (3) that the polypeptide induces antibodies that bind to SEQ ID NO: 4 **and** interfere with cell adherence in the assay described in Example 8.[1] One of skill in the art reading this disclosure clearly understands that the epitope must comply with all requirements of the claim and can be larger than 8 AA in length.

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[1] Note that the paragraph spanning specification pgs. 20-21 provides an embodiment that the useful fragments of OMP86 are characterized by the ability to induce antibodies witch interfere with binding of the pathogen to its cellular target, per the assay of Example 8, and may be as small as 5 up to fragments just less than the entire 700+ AA OMP85 protein.

Applicants submit that their disclosure describes more than simply "what the gene does". OMP85 is a minor and less abundant OMP of *N. meningitidis* or *N. gonorrhoeae*. Applicants were the first to realize the significance of the use of an immunogenic composition employing a polypeptide that can induce sufficient antibodies that bind to OMP85, and prevent the pathogen from binding to the target cell. Applicants are the first to demonstrate in the assay of Example 8 that antibodies that bind to SEQ ID NO: 4 can be shown to block pathogen-target binding. Thus, Applicants are the first to disclose the requirements of such an immunogenic composition. The importance of Applicants' disclosure of this information as of the priority date of October 22, 1998 is demonstrated by the plethora of documents published after 1998 and focusing on uses of OMP85 in the meningitis vaccine field.

The specification provides written description at page 20, lines 25-29 through to page 21, line 4 that the full-length OMP85 or fragments of these sequences representing an epitope thereof, have the ability to induce antibodies to the cellular targets of *Neisseriae*, *e.g.*, epithelial cells or mucosal cells, such as is *exemplified* in Example 8. All of this teaching, which is in the original priority specification, provides description sufficient for one of skill in the art to understand that the *N. meningitidis* and *N. gonorrhoeae* OMP85 proteins contain an epitope sequence that is recognized by the antisera developed to the exemplified *N. gonorrhoeae* OMP85.[2]

Example 8 provides written description of the use of a sequence containing an epitope found within an OMP85 amino acid sequence. In this instance, the OMP85 sequence (SEQ ID NO: 2) has at least 95% sequence similarity to SEQ ID NO: 4. Example 8 discloses the use of antisera developed to a fusion protein of the first 178 AA of SEQ ID NO: 2; the 178AA sequence differs between the two species by 3 amino acids, as shown in Fig. 5. The specification at Example 7 and Fig. 6 provides written description that the same antisera bound to OMP85 proteins of various *N. meningitidis* and *N. gonorrhoeae* strains in the Western blot. Clearly, this description conveys to one

[2] Even if every nuance of the claims is not explicitly described in the specification, the critical issue is the understanding of the skilled artisan. See, *e.g.*, Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991).

of skill in the art that antisera to amino acid 1-178 of SEQ ID NO: 2 binds to the OMP85 sequence of SEQ ID NO: 4. This is evidence that both the *N. gonorrhoeae* and *N. meningitidis* OMP85 proteins contain at least one common epitope sequence capable of inducing similarly binding antisera. This would be clearly understood by one of skill in the art considering the degree of identity of the SEQ ID NO: 2 and 4 sequences, as well as the virtual identity of the two sequences in the span of amino acids 1-178.

It is not necessary for written description of this invention to define precisely the epitope of OMP85 itself. It is sufficient for written description of this invention that Applicants have described the OMP85 protein or polypeptide that contains an epitope to which the antisera binds and that is capable of inducing the antisera for use in an immunogenic composition. One of skill in the art is aware that the identification of the precise epitope (which is a routine matter given the identification of the protein that contains it) is not necessary in order to identify a protein or polypeptide useful to induce antisera.

The description and teaching at page 20, lines 25-29 coupled with the description and teaching of Example 8 provides the essential written description to convey to the person of skill in the art that antisera to an OMP85 protein with the extremely high degree of similarity to SEQ ID NO: 4 also prevents binding between a *Neisseria* species, e.g., the *N. gonorrhoeae* species exemplified, and the known cellular target, i.e., an epithelial cell.

The specification thus clearly provides written description for the selection and use of the *Neisseria gonorrhoeae* and *Neisseria meningitidis* OMP85 proteins and fragments thereof as useful immunogens, the strong homology of the sequences between the *Neisseria gonorrhoeae* and *Neisseria meningitidis* species (described functionally and by analysis of the two exemplified amino acid sequences SEQ ID NOs: 2 and 4)[3] and the ability of these proteins or fragments thereof to induce antisera in a mammal, *e.g.*, a laboratory rabbit model, that can interfere with the ability of the *Neisseria* pathogen to

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[3] Further support can be found by comparison of the OMP85 sequence of *N. gonorrhoeae* FA1090 (GenBank No. AAW90419) shown in the specification's description and Figs. 3 and 6, with *N. meningitidis* HH OMP85. These sequences are also 95% identical.

attach to its cellular target. As the prior art at the time of filing of the priority document, was clearly aware that invasion of epithelial cells was critical to infection by *Neisseria* pathogenic species, this specification identified the value of these OMP85 proteins, as opposed to the more abundant major outer membrane proteins (e.g., PorA/PorB) known in the art for these pathogens.

Applicants incorporate the support explicitly provided in the preceding response. Given the entirety of the specification, the amendments herein which closely track language used in the specification, the supporting portions of the specification identified herein and in the preceding response, and the understanding of the skilled artisan, none of the pending, rejected claims run afoul of the written description requirement. All new and amended claim language is supported by written description in the present continuation application and its priority documents. Therefore, this rejection should be properly withdrawn.

In view of the remarks and amendments, these rejections set out in paragraphs 11-14 of the Office Action may be properly withdrawn and Claims 25, 39-42, 55 and 58 given priority of October 22, 1998.

In view of the above amendments and remarks, Applicants respectfully request that the amended claims be permitted to issue in due course.

III. 35 USC §102 Rejections

A. *Claims 50, 52, 55, 56 and 30-36 are allegedly anticipated under §102 (b) by Dunn, et al. (Microbial Pathogenesis 18:81-96, 1995) as evidenced by Mignogna, et al. (J. Proteome Res. 4:1361-1370, 2005).*

B. *Claims 50, 52, 55 and 56 are allegedly anticipated under §102 (b) by West, et al. (Infect. Immun. 47:388-394, 1985) as evidenced by Manning et al, (Microb. Pathogenesis 25:11-22, July 1998).*

Applicants respectfully request reconsideration and withdrawal of the rejections of paragraphs (A) and (B) (paragraphs 17 and 19 of the Office Action) above as against any pending claim.

Dunn 1995 discloses a typical preparation of *N.meningitidis* MS58 outer membrane vesicles, i.e., by suspending bacterial colonies in PBSB, followed by serial vortexing, centrifugations and filtering. Dunn tested the resulting OMV for toxicity on human umbilical vein endothelial cells; no information on immunogenicity was provided by this document. Mignogna 2005, which is a proteomic study that identified 210 protein species from this bacterium, identifies OMP85 as an MS58 protein. However, Applicants do not challenge the fact that OMP85 is a minor outer membrane protein of this bacterium. In fact Mignogna states that the *major* OMPs of its MS58 preparation were P.IA and P. IB (pg. 1369, col. 1, 4th full para.).

Similarly to the disclosure of Dunn, the methods and materials paragraphs of West 1985 merely recite the preparation of crude membrane preparation from the cultured *N. gonorrhoeae* FA19 bacteria grown under wild-type or iron-repressed culture conditions (see pg. 388, col. 2). Fig. 1 shows the response of FA19 to iron limitation after dilution of the culture with Desferal, an iron chelator. The arrows on the gel point to positions on the gel between the 67 and 97 kDa markers induced due to iron limitation, an unnatural culture condition. Clearly the gel indicates considerably less of the induced protein in comparison to the abundant proteins appearing on other areas of the gel in both the control and iron-limited cultures. Fig. 2, in addition to noting that the major FA19 OMP protein is between markers 30 and 43 kDA, shows essentially similar information. Further note that in col. 1 of page 391, West reports that an "additional" iron-repressible protein of 88K was present when 10μM hemoglobin was the iron source in the culture. Nothing else is stated about this protein in West or in Manning to indicate whether it is present in the crude preparation in sufficient amounts to induce anti-OMP85 antibody in a mammal.

Neither Dunn nor West can serve as §102 prior art because neither Dunn's OMV nor West's crude preparation are identical to the immunogenic composition of Applicants. Mignogna does not teach that Dunn's composition is the same as Applicants; Manning does not teach that West's composition is the same as Applicants. Neither Dunn with Mignogna nor West with Manning teach a composition with sufficient

OMP85 to *induce antibodies in a mammal that bind to said amino acid sequence of SEQ ID NO: 4 and that interfere with adherence of Neisseria gonorrhoeae as measured by the gonococcal cell adherence assay,* as **required** by Applicants' claims.

OMP85 is **not** one of the major OMPs produced in OMVs of *N. meningitidis*. Conventionally prepared OMVs (circa the 1998 priority date of this application) naturally contain much larger amounts of the major OMPs than the minor OMPs. These compositions containing abundant amounts of the major OMPs, such as PorA, would upon administration to a mammal, induce measurable antigenic responses to the major OMPs and none or undetectable immune responses to the minor proteins such as OMP85. Prior art OMVs or blebs may have contained some minor amounts of the conserved OMP85 polypeptide, but any antibody that may have developed to the minor OMPs in such compositions were swamped by antibody response to the naturally occurring, predominant OMPs. Overproduction of antisera to the predominant OMP in OMVs is one of the reasons for the failure of the prior art before 1998 to recognize the importance of OMP85 in immunogenic compositions.

Clearly the circa 1998 OMVs or crude preparations produced by centrifugation and/or detergent treatment, such as that of Dunn or West, did not contain sufficient OMP85 to elicit detectable antibodies to the minor OMP. This point is illustrated in, for example, the data of Weynants et al 2007 Infec. Immun., 75(11): 5434-5442 (attached hereto as Exhibit A), among other literature involving *N. meningitidis* or *N. gonorrhoeae* vaccines cited therein. Weynants 2007 demonstrates that animals immunized with OMVs from strains that did not overproduce OMP85 (compared to a wildtype control) produced no detectable antibodies directed against OMP85. Only when Weynants provided a recombinant bacterial strain that overproduced OMP85 did the animal's antisera produce detectable anti-OMP85 antibodies. See FIG. 2B. This data demonstrates that a conventional, non-overproducing OMV, such as provided by Dunn or West, would not have contained sufficient OMP85 to elicit detectable antibodies to SEQ ID NO: 4 and thus interfere with cellular adhesion. Applicants by virtue of Example 8

provided evidence that such anti-SEQ ID NO: 4 antibodies have a role in interfering with cellular attachment necessary for infection by *N. meningitidis* or *N. gonorrhoeae*.

In contrast to the crude OMV preparations of Dunn or West, Applicants' immunogenic composition is required to have sufficient OMP85 to induce an antibody response measurable on the cellular adhesion assay of Example 8. Thus neither Dunn's nor West's wild-type OMVs anticipate Applicants' claimed compositions. As all claims contain the requirement that the composition be able to induce antibodies to SEQ ID NO: 4 (OMP85) and are active in the assay, and further in view of these remarks, these rejections of paragraphs (A) and (B) may be withdrawn as against pending claims 50, 52, 55, 56 and 30-36 or against any claim dependent thereupon.

> ***C.*** *Claims 25, 39-42, 44-46, 55, 56 and 58 are allegedly anticipated under §102 (b) by Manning et al, (Microb. Pathogenesis 25:11-22, July 1998) in light of Richarme, et al. (Ann. Microbiol. 133A:199-204, 1982). The examiner applies this rejection because the examiner asserts that Applicants' pending claims are not entitled to their priority date and are permitted only the date of June 26, 2003, thereby making this document a basis for prior art rejection.*

Applicants respectfully request reconsideration and withdrawal of this rejection as against any pending claim.

The pending specification is a continuation of the prior application filed October 22, 1998 and has the same specification with minor formal and grammatical corrections. Manning, a publication of the inventors[4], published less than one year prior to the priority date, is not §102(b) prior art for any claim entitled to claim priority to the original application.

In view of the claim amendments and remarks provided above in Para. II and cancellation of Claims 44-46, amended Claim 25, dependent claims 39-42, and Claims *55, 56 and 58* are believed to no longer be subject to the new matter or written description rejections. These claims are thereby entitled to the priority date of October 22, 1998 and are free of this rejection as set out in paragraph 9 of the Office Action.

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[4] The previously filed *In re Katz* declaration removes Manning from citation as §102(a) prior art.

Applicants respectfully request reconsideration and withdrawal of this rejection

D. *Claims 25, 39-42, 44-46 and 55 are rejected as allegedly anticipated under §102(e)(2) by US 6,551,795 (Rubenfield) as evidenced by Harlow et al, in Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, pp.471-510 (1988). Rubenfield discloses an isolated or substantially pure P. aeruginosa 648 amino acid polypeptide having an amino acid sequence comprising the eight consecutive amino acids VRVETADG which are identical to an 8-mer of the instant SEQ ID NO: 4. Rubenfield's polypeptide is employed in a vaccine composition, a diagnostic composition, a diagnostic reagent, a kit, etc.*

Applicants respectfully request reconsideration and withdrawal of this rejection as against any pending claim.

In view of the cancellation of Claims 44-46, and amendment of Claim 25, Claim 25 and its dependent claims 39-42 are no longer subject to this rejection. These claims no longer refer to an epitope of unspecified length. Any alleged overlap with the disclosure of Rubenfield is removed.

With regard to Claim 55, the claim does not cover any stretch of 8 amino acids that can be found within SEQ ID NO: 4, but instead requires that (1) the polypeptide is isolated; (2) that the polypeptide comprise an epitope of *at least* 8AA of SEQ ID NO: 4; ***and*** (3) that the polypeptide induces antibodies that bind to SEQ ID NO: 4 **and** interfere with cell adherence in the assay described in Example 8.[5] One of skill in the art reading this disclosure clearly understands that the epitope must comply with all requirements and can be larger than 8 AA in length. Rubenfield in no way discloses that the 8-mer identified *by the examiner* in its *P. aeruginosa* sequence meets any of Applicants' requirements, other than a minimal length. Specifically, Rubenfield's 8-mer is not isolated or identified as relevant in any way by the Rubenfield inventors. There is no evidence that Rubenfield's 8-mer induces antibodies to SEQ ID NO: 4; nor is there evidence or teaching that this 8-mer induces antibodies that interfere with binding of a Neisserial pathogen to its cellular target.

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[5] Note that the paragraph spanning specification pg 20-21 provides an embodiment that the useful fragments of OMP85 are characterized by the ability to induce antibodies which interfere with binding of the pathogen to its cellular target, per the assay of Example 8, and may be as small as 5 up to fragments just less than the about 790 AA OMP85 protein.

As Applicants were the first to realize the significance of the use of an immunogenic composition employing a polypeptide that can induce sufficient antibodies that bind to OMP85 of *N. meningitidis* or *N. gonorrhoeae* and are active in preventing the pathogen from binding to the target cell in the assay of Example 8, they are the first to disclose the requirements of such an immunogenic composition. No such disclosure or recognition is found in Rubenfield, which is directed to a completely different pathogen.

This rejection as set out in paragraph 16 of the Office Action may properly be withdrawn.

> ***E.*** *Claims 25, 39-42 and 44-46 are allegedly anticipated under §102 (b) by Chong, et al. (WO 94/12641) as evidenced by Harlow et al, in Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, pp.471-510 (1988). Chong discloses a 27 AA fragment of which 6 AA are found contiguously in SEQ ID NO: 4. "Therefore, the prior art polypeptide of SEQ ID NO: 35 is viewed as an epitope of the instantly recited SEQ ID NO: 4."*

Applicants respectfully request reconsideration and withdrawal of this rejection as against any pending claim. Cancellation of claims 44-46 moots this rejection as to them. In no way would one of skill in the art consider a 27-mer polypeptide sharing only 6 amino acids (DGVSLG) in common with SEQ ID NO: 4 be considered an epitope of Applicant's SEQ ID NO: 4. As more than half of Chong's SEQ ID NO: 35 is not identical with Applicants' SEQ ID NO: 4, and Chong's stated intention was that its polypeptides and proteins were to be the useful immunogenic polypeptide and diagnostic reagent for *H. influenzae*, not for *N. meningitidis* or *N. gonorrhoeae*, Applicants submit that this rejection should be withdrawn.

This rejection as set out in paragraph 18 of the Office Action may properly be withdrawn.

In summary, in view of the remarks and amendments above:

- Claim 57 is in allowable form, as it was subject to no rejections;
- Claims 50, 52 and 32-36 are allowable as they are free of the double-patenting rejection due to the terminal disclaimer, and free of the rejections over Dunn and/or West;
- Claim 58 is allowable as it is free of the §112 rejections and entitled to the priority date, and thus free of the §102(b) rejection over Manning;
- Claims 25, 39-42, and 55 are allowable as they are free of the §112 rejections and entitled to the priority date, and thus free of the §102(b) rejection over Manning; and are further patentable over §102(e)(2) rejection over Rubenfield, and the §102(b) rejection over Chong.

Applicants have responded to all rejections stated in the outstanding Office Action and submit that the examiner may properly withdraw all rejections and allow this application to proceed to issuance.

The Director is hereby authorized to charge any deficiency in any fees due with the filing of this paper or during the pendency of this application, or credit any overpayment in any fees to our Deposit Account Number 08-3040.

Respectfully submitted,

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Exhibit A

INFECTION AND IMMUNITY, Nov. 2007, p. 5434–5442
0019-9567/07/$08.00+0 doi:10.1128/IAI.00411-07

Vol. 75, No. 11

Additive and Synergistic Bactericidal Activity of Antibodies Directed against Minor Outer Membrane Proteins of *Neisseria meningitidis*[▽]

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Received 20 March 2007/Returned for modification 1 May 2007/Accepted 16 July 2007

***Neisseria meningitidis* serogroup B is a major cause of bacterial meningitis in younger populations. The available vaccines are based on outer membrane vesicles obtained from wild-type strains. In children less than 2 years old they confer protection only against strains expressing homologous PorA, a major, variable outer membrane protein (OMP). We genetically modified a strain in order to eliminate PorA and to overproduce one or several minor and conserved OMPs. Using a mouse model mimicking children's PorA-specific bactericidal activity, it was demonstrated that overproduction of more than one minor OMP is required to elicit antibodies able to induce complement-mediated killing of strains expressing heterologous PorA. It is concluded that a critical density of bactericidal antibodies needs to be reached at the surface of meningococci to induce complement-mediated killing. With minor OMPs, this threshold is reached when more than one antigen is targeted, and this allows cross-protection.**

Infectious diseases caused by *Neisseria meningitidis* are a significant public health concern. *N. meningitidis* serogroup B (MenB) caused 69% of meningococcal disease reported in Europe in 2004 (10). MenB has also caused outbreaks in several countries with annual attack rates of 5 to 50 cases per 100,000 persons, with most cases occurring in young children (5). Overall, MenB causes a substantial proportion of diseases across all ages, but the specific distribution varies by age group, with higher proportions in infants and toddlers than in older age groups (27, 33). Conjugate polysaccharide vaccines based on the capsular polysaccharide of *N. meningitidis* serogroups A, C, W-135, and Y have been licensed for adolescents, and pediatric development is ongoing. However, utilization of the serogroup B capsular polysaccharide as a vaccine antigen has been hampered by its poor immunogenicity and by potential concern about inducing autoantibodies that cross-react with glycosylated host antigens (11, 26). Alternative antigens are therefore being evaluated as candidates for use in a vaccine against MenB strains.

It is possible to extract the outer membrane from *N. meningitidis* or culture supernatant in the form of outer membrane vesicles (OMVs). Vaccines based on OMVs have been developed by using detergent extraction to reduce the lipooligosaccharide (LOS) content (13). PorA is one of the most abundant outer membrane proteins (OMPs) displaying high antigenic variability, which is used to classify meningococci (14). OMV vaccines made from single wild-type strains induce protection in children more than 4 years old in a PorA serosubtype-independent way (8). In children less than 2 years old, wild-type OMV vaccines predominantly induce PorA serosubtype-specific serum bactericidal activity (29, 41, 46). Efforts to develop cross-protective vaccines, especially in younger populations, are ongoing (32).

Ideally, a vaccine to prevent MenB disease should be safe and immunogenic in the pediatric population and elicit protection against a wide range of clinical isolates (34). In this context, we are actively pursuing the development of a multicomponent vaccine containing conserved surface antigens able to induce cross-protective immune responses. In order to limit the risk of the appearance of vaccine escape mutants, our research is oriented towards a vaccine able to interfere with several mechanisms of the meningococcal infectious process, such as iron uptake (39), toxicity (42), and adhesion (4).

To overcome limitations of recombinant expression and folding of integral OMPs, an alternative expression system in *N. meningitidis* was developed by taking into account the capacity of this organism to produce large amounts of OMVs in the presence of detergent. Overproduction of OMPs that might have potential as vaccine antigens was achieved by using two methodologies referred to as gene delivery and promoter replacement (35). When the overexpressed gene encodes a surface component, the resulting recombinant strain produces OMVs enriched in the desired component.

In the present study, four minor OMPs (TbpA, Hsf, NspA, and Omp85) that have already shown some potential as vaccine candidates, being surface exposed and well conserved among serogroup B neisseria strains, were overexpressed. TbpA is an integral OMP that, together with TbpB, makes up the transferrin receptor of *N. meningitidis* (21, 30, 36, 40). Sera from carriers and subjects with meningococcal diseases, but

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▽ Published ahead of print on 30 July 2007.

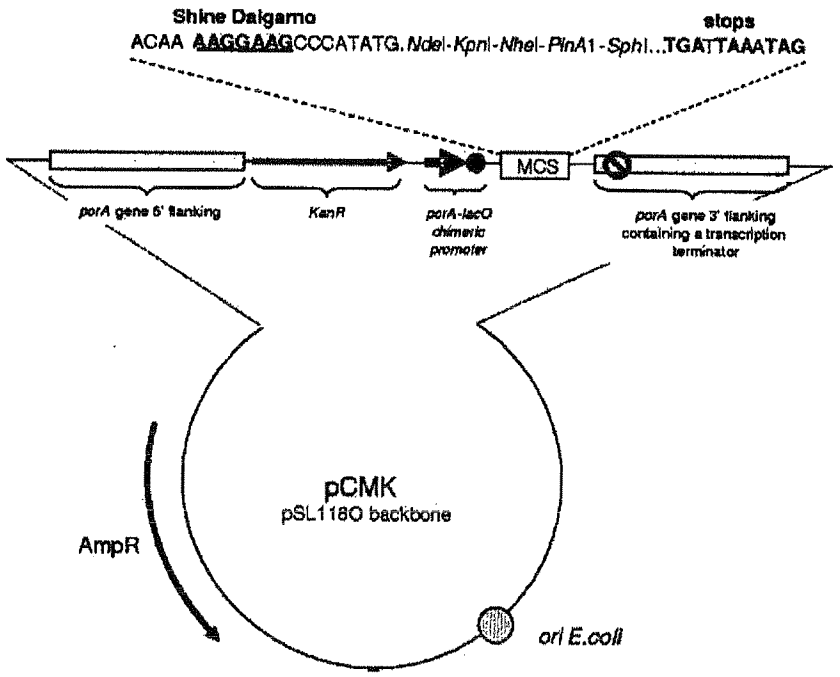


FIG. 1. Schematic representation of the pCMK vectors used to deliver genes, operons, and/or expression cassettes in the genome of *N. meningitidis*. MCS, multiple cloning site.

not sera from controls, had detectable antibodies to TbpA/B, suggesting that there is expression of TbpA/B by *Neisseria* in vivo (1, 18). Affinity-isolated Tbp proteins from *N. meningitidis* induced protection against challenge in mice after passive or active immunization (7). Omp85 is a minor antigen present in *N. meningitidis* and in OMVs (22, 28). Omp85 is highly conserved (12) and is an essential protein involved in the positioning and folding of other OMPs in the bacterial outer membrane (3, 17, 45). There is a correlation between the presence of antibodies against an 80-kDa protein detected by Western blotting and bactericidal activity after immunization with wild-type OMV vaccine (37). Hsf (or NhhA) is the neisserial autotransporter protein homologous to *Haemophilus influenzae* Hsf/Hia. Hsf is well conserved among *N. meningitidis* strains, its gene has been detected in all strains tested, and the protein is surface located (31). It has been suggested that Hsf acts as an adhesin (38). Recombinant Hsf is also recognized by human serum from patients and carriers (44). NspA is present on the surface of 99% of meningococcal strains tested and is well conserved (24). Immunization of mice with NspA induced protection against *N. meningitidis* challenge (23). Based on sequence similarity with Opa proteins, it is possible that NspA is an adhesin (43).

We developed a mouse model mimicking the PorA-specific bactericidal response observed in younger populations with wild-type OMVs. To overcome the PorA-restricted protection of wild-type OMVs, expression of PorA in the H44/76 strain was suppressed, and to avoid the presence of the polysaccharide B capsule, the *cps* gene complex was deleted. This deletion also removed the *galE* gene, resulting in the synthesis of truncated LOS. Using the *porA galE*, acapsulate mutant strain as the expression host, we evaluated the impact on the vaccine response of overproduction of four minor well-conserved OMPs (TbpA, Hsf, NspA, and Omp85) for which a potential role in protection has been demonstrated.

MATERIALS AND METHODS

***N. meningitidis* transformation.** Cells of *N. meningitidis* strain H44/76 (B:15:P1.7,16:L3,7) incubated overnight in the presence of 5% CO_2 on chocolate base (GC) (Difco) or Mueller-Hinton (MH) (Difco) medium plates were collected in 2 ml of liquid GC or MH medium containing 10 mM $MgCl_2$ and diluted to obtain an optical density at 550 nm (OD_{550}) of 0.1. Two micrograms of DNA was added to the cell suspension, and this was followed by a 6-h incubation at 37°C (with shaking). After the incubation period, 100 μl of the culture, undiluted or diluted 1/10, 1/100, or 1/1,000, was spread on GC or MH medium plates containing the appropriate antibiotic (see below). Recombinant colonies appeared after 48 h of incubation at 37°C in the presence of 5% CO_2.

Construction of H44/76 lacking capsular polysaccharides (*cps*). Plasmid pMF121 (16) was used to construct an H44/76 derivative lacking the capsular polysaccharide. This plasmid contains the flanking regions of the gene locus coding for the biosynthesis pathway of the group B polysaccharide and an erythromycin resistance gene. Deletion of the group B polysaccharide locus resulted in loss of expression of the group B capsular polysaccharide and loss of the active copy of the *galE* gene, leading to galactose-deficient LOS. Erythromycin (10 μg/ml)-resistant colonies were selected, and capsule-deficient strains were identified by colony blotting using the anti-group B polysaccharide 735 monoclonal antibody (DadeBehring, Marburg, Germany). Binding of the monoclonal antibody was visualized with a biotinylated anti-mouse immunoglobulin (1/1,000; Amersham).

Construction of plasmid pCMK, targeting integration in the *porA* locus of H44/76. A schematic drawing of the pCMK vector is presented in Fig. 1. pCMK is a high-copy-number plasmid that replicates in *Escherichia coli*, was derived from a pSL1180 backbone (PharmaciaBiotech), and harbors the *bla* gene, thereby conferring resistance to ampicillin. In addition, pCMK contains two *porA* flanking regions (porA5′ and porA3′ containing a transcription terminator) necessary for homologous recombination, a selectable marker conferring resistance to kanamycin, two uptake sequences, a *porA/lacO* chimeric promoter

TABLE 1. Primers used in this study

Primer	Nucleotide sequence[a]	Relevant characteristic(s)
PorA5'Fwd	5'-CCCAAGCTTGCCGTCTGAATACATCCCGTCATTCCTCA-3'	HindIII, uptake sequence
PorA5'Rev	5'-CGATGCTCGCGACTCCAGAGACCTCGTGCGGGCC-3'	NruI
PorA3'Fwd	5'-GGAAGATCTGATTAAATAGGCGAAAATACCAGCTACGA-3'	BglII, stop codons
PorA3'Rev	5'-GCCGAATTCTTCAGACGGCGCAGCAGGAATTTATCGG-3'	EcoRI, uptake sequence
PorAlcOFwd	5'-AAGCTCTGCAGGAGGTCTGCGCTTGAATTG-3'	PstI
PorAlacORev	5'-CTTAAGGCATATGGGCTTCCTTTTGTAA-3'	NdeI
PPA1	5'-GCGGCCGTTGCCGATGTCAGCC-3'	
PPA2	5'-GGCATAGCTGATGCGTGGAACTGC-3'	
N01-full-NdeI	5'-GGGAATTCCATATGAAAAAAGCACTTGCCACAC-3'	NdeI
NdeI-NspA 3	5'-GGAATTCCATATGTCAGAATTTGACGCGCAC-3'	NdeI
HSF 01-NdeI	5'-GGAATTCCATATGATGAACAAAATATACCGC-3'	NdeI
HSF 02-NheI	5'-GTAGCTAGCTAGCTTACCACTGATAACCGAC-3'	NdeI
ProD15-51X	5'-GGGCGAATTCGCGGCCGCCGTCAACGGCACACCGTTG-3'	EcoRI
ProD15-52	5'-GCTCTAGAGCGGAATGCGGTTTCAGACG-3'	XbaI
TnRD15-KpnI/XbaI	5'-CGCCGGTACCTCTAGAGCCGTCTGAACCACTCGTGGACAACCC-3'	KpnI and XbaI, uptake sequence
TnR03Cam(KpnI)	5'-CGCCGGTACCGCCGCTAACTATAACGGTC-3'	KpnI
PorA-01	5'-CGCCGGTACCGAGGTCTGCGCTTGAATTGTG-3'	KpnI
PorA02	5'-CGCCGGTACCTCTAGACATCGGGCAAACACCCG-3'	KpnI
BAD16	5'-GGCCTAGCTAGCCGTCTGAAGCGATTAGAGTTTCAAAATTTATTC-3'	NheI, uptake sequence
BAD17	5'-GGCCAAGCTTCAGACGGCGTTCGACCGAGTTTGAGCCTTTGC-3'	HindIII, uptake sequence
BAD18	5'-TCCCCCGGGAAGATCTGGACGAAAAATCTCAAGAAACCG-3'	XmaI and BglII
BAD19	5'-GGAAGATCTCCGCTCGAGCAAATTTACAAAAGGAAGCCGATATGCA ACAGCAACATTTGTTCCG-3'	BglII and XhoI
BAD21	5'-GGAAGATCTCCGCTCGAGACATCGGGCAAACACCCG-3'	BglII and XhoI
BAD20	5'-TCCCCCGGGAGATCTCACTAGTATTACCCTGTTATCCC-3'	XmaI, BglII, and SpeI

[a] Restriction sites are in bold type; uptake sequences or stop codons are underlined.

repressed in the *E. coli* host [BL21(DE3)] expressing *lacI*^{q} but transcriptionally active in *N. meningitidis*, and a multiple cloning site (with five sites: NdeI, KpnI, NheI, PinAI, and SphI) necessary for insertion of foreign DNA into pCMK.

The *porA5'* and *porA3'* recombinogenic regions and the *porA/lacO* promoter were PCR amplified from genomic DNA extracted from H44/76 using oligonucleotides PorA5'Fwd, PorA5'Rev, PorA3'Fwd, PorA3'Rev, PorAlacORev, and PorAlacOFwd (Table 1) under the conditions described by the supplier of HiFi DNA polymerase (Boehringer, Mannheim, Germany) and cloned in pSL1180. The kanamycin resistance cassette was excised from pUC4K (PharmaciaBiotech) by PstI restriction and introduced between the porA5' flanking region and the *porA/lacO* promoter region.

Construction of an H44/76 Δ*porA* strain. The H44/76 *cps* strain was transformed with 2 μg of supercoiled pCMK plasmid DNA as described above and plated on kanamycin-containing plates (200 μg/ml). Kanamycin-resistant colonies were screened for deletion of the *porA* gene by PCR with boiled bacterial lysates using primers PPA1 and PPA2 (Table 1). The absence of PorA synthesis was further confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis.

Overproduction of NspA and Hsf in H44/76 (gene delivery). The gene coding for NspA was PCR amplified from genomic DNA extracted from H44/76 using the N01-full-NdeI and NdeI-NspA 3 oligonucleotide primers containing NdeI restriction sites (Table 1). The corresponding amplicon was digested with NdeI and inserted into the NdeI restriction site of the pCMK delivery vector.

The gene coding for Hsf was PCR amplified from genomic DNA extracted from H44/76 using the HSF 01-NdeI and HSF 02-NheI oligonucleotide primers (Table 1). Because of the sequence of the HSF 01-NdeI primer, the Hsf protein produced contained two methionine residues at the N terminus. The corresponding amplicon was subsequently cloned in the NdeI restriction site of the pCMK delivery vector. In the recombinant plasmid, designated pCMK-Hsf, we deleted the *lacO* gene present in the chimeric *porA/lacO* promoter.

Two micrograms of pCMK-NspA or pCMK-Hsf was used to transform the H44/76 Δ*cps* strain. Kanamycin-resistant colonies were screened for deletion of the *porA* gene and insertion of a second copy of the *nspA* gene or the *hsf* gene by PCR using boiled bacterial lysates. The absence of PorA synthesis and overproduction of NspA or Hsf were further confirmed by SDS-PAGE analysis.

Overproduction of Omp85 in H44/76 (promoter delivery). A promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA fragment covering nucleotides −983 to −48 with respect to the *omp85* gene start codon (ATG) was PCR amplified from genomic DNA extracted from the H44/76 strain using oligonucleotides ProD15-51X and ProD15-52 containing EcoRI and XbaI restriction sites, respectively (Table 1). This fragment was subjected to restriction and inserted into the pUC18 plasmid (PharmaciaBiotech) restricted with the same enzymes. The construct that we obtained was subjected to in vitro mutagenesis using the genome priming system (with the pGPS2 donor plasmid) commercialized by New England Biolabs. Clones in which a mini-transposon (derived from Tn*7* and harboring a chloramphenicol resistance gene) was inserted were selected. One clone containing a mini-transposon insertion located in the *omp85* 5' flanking region, 401 bp downstream from the EcoRI site, was isolated and used for further studies. This plasmid was subjected to circle PCR mutagenesis in order to (i) delete a repeated DNA sequence (Tn*7R*) generated by the transposition process, (ii) insert meningococcal uptake sequences required for transformation, and (iii) insert suitable restriction sites allowing cloning of foreign DNA material, such as promoters. The circle PCR was performed using the TnRD15-KpnI/XbaI and TnR03Cam(KpnI) oligonucleotides containing uptake sequences and restriction sites (KpnI and XbaI) (Table 1). The resulting PCR fragment was gel purified, digested with Asp718 (isoschizomer of KpnI), and ligated to a 184-bp DNA fragment containing the *porA* promoter and generated by PCR using the PorA-01 and PorA02 oligonucleotides containing KpnI restriction sites. A recombinant plasmid (pUC OMP85) carrying a *porA* promoter inserted in the correct orientation was selected and used to transform H44/76 lacking capsular polysaccharide (Δ*cps*) and PorA (Δ*porA*). Recombinant H44/76 clones resulting from a double-crossover event (PCR screening) were selected on GC medium containing 5 μg/ml chloramphenicol and analyzed for Omp85 synthesis.

Overproduction of TbpA in H44/76 (promoter delivery). The *tbpB* gene was deleted and replaced by the Cm^r/PorA promoter cassette. A 3,218-bp DNA fragment corresponding to the 509-bp 5' flanking region of the *tbpB* gene, the 2,139-bp *tbpB* coding sequence, the 87-bp intergenic sequence, and the first 483 nucleotides of the *tbpA* coding sequence was PCR amplified from H44/76 genomic DNA using oligonucleotides BAD16 and BAD17 containing uptake sequences and NheI and HindIII restriction sites (Table 1). This PCR fragment was cloned in a pGEM-T vector (Promega). The plasmid was subjected to circle PCR mutagenesis in order to (i) insert suitable restriction sites allowing cloning of a Cm^r/PorA promoter cassette and (ii) delete 209 bp of the 5' flanking sequence of *tbpB* and the *tbpB* coding sequence. The circle PCR was performed using the BAD18 and the BAD19 oligonucleotides containing XmaI, BglII, and XhoI restriction sites (Table 1). The Cm^r/PorA promoter cassette was amplified from the pUC OMP85 plasmid using primers BAD21 and BAD20 containing

TABLE 2. Summary of invasive *N. meningitidis* strains used in bactericidal assays

Strain	Country of origin	Year isolated	PorA classification	Epidemic strain
H44/76	Norway	1976	P1.7,16	Yes
CU385	Cuba	1980	P1.19,15	Yes
M97-250687	United Kingdom	1997	P1.19,15	No
NZ124/98	New Zealand	1998	P1.7,4	Yes

XmaI, SpeI, BglII, and XhoI restriction sites (Table 1). This PCR fragment was cloned in the circle PCR plasmid. Two micrograms of this plasmid was used to transform the H44/76 *Δcps ΔporA* strain. Integration by double crossover in the upstream region of *tbpA* directed insertion of the *porA* promoter directly upstream of the *tbpA* start codon. Recombinant H44/76 clones resulting from a double-crossover event (PCR screening) were selected on GC medium containing 5 μg/ml chloramphenicol and analyzed for TbpB down-expression and TbpA synthesis.

Culture and preparation of OMVs. A vial of frozen *N. meningitidis* (recombinant or not recombinant) was thawed and streaked onto a modified Frantz agar plate, which was then incubated at 37°C for 18 h. Colonies were resuspended, added to a flask containing modified Frantz medium supplemented with the appropriate antibiotic, and incubated at 37°C for 16 h with shaking. The cells were separated from the culture broth by centrifugation at 5,000 × *g* at 4°C for 15 min. OMVs were isolated using deoxycholate as described previously (15).

SDS-PAGE. OMV preparations were analyzed by SDS-PAGE. After heating for 5 min at 100°C in sample buffer, 15 μg was loaded onto the gel. After electrophoresis, gels were stained with Coomassie brilliant blue R250.

Mice and immunizations. Outbred OF1 mice (female; 6 to 8 weeks old; also known as CF1; Charles River, Lyon, France) received three injections with OMVs via the intramuscular route on days 0, 21, and 28. With each 50-μl injection, 5 μg of antigen formulated in the GSK proprietary AS04 adjuvant ($AlPO_4$ plus 3-*O*-deacyl-4′-monophosphoryl lipid A) was administered. Control mice received only adjuvant. Blood samples were collected 14 days after the third injection. The experiments complied with the relevant national guidelines of Belgium and institutional policies of GlaxoSmithKline Biologicals.

Antibody assays. Omp85 and TbpA derived from strain H44/76 were expressed without their signal sequence in *E. coli*, where they accumulated in inclusion bodies. These bodies were purified and solubilized as described previously (20). The passenger domain of Hsf derived from strain H44/76 was expressed and purified from *E. coli* as a C-terminally His-tagged protein. NspA derived from strain B11 was expressed without its signal sequence and was purified from *E. coli* as an N-terminally His-tagged protein.

Enzyme-linked immunosorbent assay (ELISA) plates were coated with TbpA, Hsf, NspA, or Omp85 in phosphate-buffered saline (PBS). The assays were performed as described previously (20).

Complement-dependent bactericidal antibody assays. Wild-type MenB strains used in this study were isolated either from cases during epidemics occurring in different regions around the world (epidemic strains) or from a case isolate in the United Kingdom (nonepidemic strain) (Table 2). The strains were grown overnight on MH agar (Difco) containing 1% (vol/vol) Polyvitex (Biomérieux) and 1% horse serum (Sigma) at 37°C in a 5% CO_2 atmosphere. The bacteria were inoculated into tryptic soy broth (Becton Dickinson) with 50 μM of the iron chelator desferrioxamine mesylate (Sigma) and were grown in shaking flasks for 3 h at 37°C until an OD_{470} of 0.5 was reached. Each culture was then diluted in PBS containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 0.1% glucose (PBS-glucose) in order to obtain an OD_{600} of 0.4 (bacterial suspension). The sera were heat inactivated (40 min at 56°C) and subsequently diluted 1/50 in PBS-glucose. In wells of sterile flat-bottom microtiter plates (Nunc), 25 μl of diluted test serum was mixed with 12.5 μl of baby rabbit complement (selected for the absence of bactericidal activity against the test strains; Cerdarlane Laboratories) and 12.5 μl of the bacterial suspension. Serial dilutions of test sera were treated similarly. The controls included bacteria plus complement, bacteria plus heat-inactivated complement, and test serum plus bacteria plus heat-inactivated complement. Antiserum from mice immunized three times with whole bacterial cells (5 μg of protein with AS04 per injection) was used as a positive control in the bactericidal assays and also to validate or reject each microtiter plate. The microtiter plates were then sealed and incubated with shaking (520 rpm) for 75 min at 37°C without CO_2. After this incubation, 50 μl of MH medium containing 0.9% agar was added to each well. A second layer of 50 μl of PBS containing 0.9% agar was added 30 min later. After overnight incubation at 33°C in the presence of 5% CO_2, the colonies were counted. The average number of CFU in the controls corresponding to bacteria plus complement was defined as 100%. The bactericidal titer was defined as the reciprocal of the serum dilution that resulted in 50% killing.

TABLE 3. Complement-mediated bactericidal titers of sera from mice immunized with PorA$^+$ OMV vaccines[a]

Vaccine	Bactericidal activity with strain[b]:			
	H44/76 (P1.7,16)	Cu385 (P1.19,15)	M97-250687 (P1.19,15)	NZ124/98 (P1.7,4)
P1.7,16 OMVs	1,568	<100	<100	<100
P1.19,15 OMVs	<100	1,082	870	<100

[a] The bactericidal activity is expressed as the reciprocal dilution of serum required to kill 50% of bacteria. The assay was performed with pooled sera from 10 mice per group.

[b] The PorA serosubtypes of the strains are indicated in parentheses.

Possible additive or synergistic effects of antibodies directed against different overexpressed minor OMPs were studied by using several pooled sera. Pools were obtained from sera derived from 20 mice after immunization with the same vaccine preparation. For mixing experiments, equal volumes of pools from the same treatment group were combined and subsequently tested in the bactericidal assay.

Statistical analysis. Differences in bactericidal antibody titers were determined by the Kruskal-Wallis nonparametric test with the one-tailed Dunn test. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

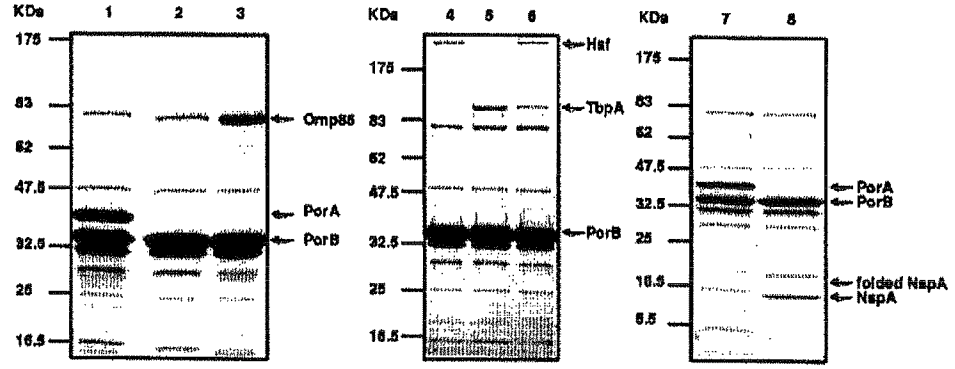
Relevance of the mouse model to the clinical situation of infants. OMVs prepared from wild-type strains H44/76 and Cu385 and formulated in the AS04 Adjuvant System were administered to OF1 mice (10 animals per group) via the intramuscular route at 0, 21, and 28 days. Serum samples were obtained 2 weeks after the third injection, pooled (10 sera per group), and tested for their bactericidal activity against four strains isolated from cases in different countries around the world. These strains expressed PorA which was either homologous or heterologous to the PorA of the vaccine strains.

Pooled antisera prepared from mice immunized with OMVs obtained from H44/76 producing P1.7,16 PorA were bactericidal against strain H44/76 but not against the heterologous PorA strains Cu385, M97-250687, and NZ124/98 (Table 3). Similar observations were made with pooled antisera from mice immunized with OMVs from strain Cu385 producing P1.19,15 PorA, which were bactericidal against the Cu385 and M97-250687 strains but not against heterologous PorA strains (H44/76 and NZ124/98). Control sera from mice immunized with AS04 alone exhibited no or undetectable bactericidal activity against the four strains. Positive control sera were obtained from mice immunized with the corresponding heat-inactivated whole bacteria (data not shown).

These results demonstrate that the immunogenicity of OMVs in OF1 mice reflects the immunogenicity of wild-type OMV vaccines in human infants; i.e., they predominantly induce a PorA serosubtype-specific serum bactericidal activity (41).

Overproduction of minor OMPs. To avoid a predominant bactericidal response directed against PorA, we decided to analyze the impact of overproduction of minor OMPs in a *porA* knockout background. Similarly, to further increase vaccine

A



B

Upregulated OMPs	Methodology	ELISA titers after immunization with Upregulated OMVs	Control OMVs	AS04
TbpA	PR	1,600	<50	<50
Hsf	GD	51,200	<50	<50
NspA	GD	400	<50	<50
Omp85	PR	200	<50	<50
TbpA/Hsf	PR/GD	400/51,200	<50	<50

FIG. 2. Overproduction of Omp85, Hsf, TbpA, and NspA. (A) Impact on the content of proteins in MenB OMVs. OMVs purified from different *N. meningitidis* strains were separated by SDS-PAGE and stained with Coomassie brilliant blue. The strains used are wild-type strain H44/76 (lanes 1 and 7), a *galE porA* mutant derivative of H44/76 (lane 2), and *galE porA* mutants overexpressing Omp85 (lane 3), Hsf (lane 4), TbpA (lane 5), Hsf and TbpA simultaneously (lane 6), or NspA (lane 8). In lane 8, bands corresponding to denatured and nondenatured NspA are present. (B) Impact on the induction of antibodies against minor OMPs in mice immunized with OMVs purified from either overproducing strains or control strains or in mice immunized with adjuvant alone (AS04) (20 mice per group). Overproduction was achieved by either the promoter replacement (PR) or gene delivery (GD) strategy. ELISA titers are expressed as the reciprocal dilutions of pooled sera required to obtain an OD_{490} of 0.5, using purified recombinant TbpA, Hsf, NspA, or Omp85.

specificity and prevent unwanted responses, such as the production of bactericidal antibodies directed against LOS, a *galE* mutant background was used.

Two different strategies were used to overproduce minor OMPs. TbpA and Omp85 were overproduced by promoter replacement, while Hsf and NspA were overproduced by gene delivery. In both cases, the *porA* promoter was selected for overexpression.

The impact of overproduction of minor OMPs on their amounts in OMVs prepared from recombinant strains was determined by SDS-PAGE. PorA was not present in OMVs purified from recombinant strains (Fig. 2A, lanes 2, 3, 4, 5, 6, and 8). Compared to wild-type OMVs (lanes 1 and 7), the levels of Omp85 (lane 3), Hsf (lanes 4 and 6), TbpA (lanes 5 and 6), and NspA (lane 8) were clearly enhanced in OMVs from the genetically modified strains. We were also able to overproduce simultaneously two different OMPs (lane 6). However, the double overproduction of TbpA and Hsf in a single strain reduced the level of TbpA compared to the level when it was overproduced alone (lanes 5 and 6), whereas the level of Hsf was not affected (lanes 4 and 6).

Thus, overproduction of TbpA, Hsf, NspA, and Omp85 by gene delivery or promoter replacement resulted in significant increases in the amounts of these proteins in OMVs.

Impact of overproduction of minor OMPs on antibody responses. Groups of 20 mice were immunized intramuscularly with different OMV preparations (5 μg protein per injection) formulated with AS04. Serum samples were obtained 2 weeks after the third dose and pooled. In ELISA, pooled sera from control mice inoculated with adjuvant alone or from mice immunized with OMVs from a nonoverproducing strain had no detectable antibodies directed against TbpA, Hsf, NspA, and Omp85 (titers, <50), whereas immunization of mice with OMVs prepared from the strain that overproduced minor OMPs elicited the production of antibodies against the respective OMPs (Fig. 2B). The most impressive increase was observed with Hsf (at least a 1,024-fold increase). The lower overproduction of TbpA in H44/76 overexpressing both *tbpA* and *hsf* affected the anti-TbpA response (titer, 400 versus 1,600).

Antibodies induced via TbpA and Hsf overproduction show additive effects in complement-mediated bactericidal activity. The serum samples obtained from mice immunized with different OMV preparations were tested individually in serum

TABLE 4. Impact of upregulation of minor OMPs on the induction of complement-mediated killing by bactericidal antibodies in mice

porA knockout *galE* OMV vaccine	MenB strain H44/76			MenB strain Cu385			MenB strain NZ124/98		
	Geometric mean titer for 50% killing[a]	95% Confidence interval	Seroconversion (%)[b]	Geometric mean titer for 50% killing	95% Confidence interval	Seroconversion (%)[b]	Geometric mean titer for 50% killing	95% Confidence interval	Seroconversion (%)[b]
No upregulation	71	46–109	15	59	42–82	5	50	50–50	0
TbpA upregulated	134	61–296	30	117	57–241	30	85	48–149	20
Hsf upregulated	74	45–122	15	73	44–121	15	88	52–149	25
TbpA and Hsf upregulated	727	342–1,546	90	244	117–511	65	222	91–542	45

[a] The geometric mean titer for 50% killing was calculated with individual sera ($n = 20$).
[b] Percentage of responder mice (titer, >50).

bactericidal assays against three MenB strains (20 mice per group). These strains included parental wild-type strain H44/76 and heterologous strains Cu385 and NZ124/98. Vaccine preparations obtained from *porA galE* mutant strains either not overproducing OMPs (control vaccine) or overproducing Hsf alone elicited low or undetectable serum bactericidal antibody titers in mice (geometric mean titer, ≤88) with a low percentage of seroconversion (from 0 to 25% of mice developed detectable bactericidal antibodies) (Table 4). The serum bactericidal titers against H44/76 or Cu385 were slightly higher when tests were performed with sera from mice immunized with OMVs from the TbpA-overproducing strain, and the seroconversion rates were also slightly higher. However, the bactericidal responses elicited by the control vaccine and the single-OMP-overproducing-strain vaccines were not statistically different. In contrast, mice immunized with OMVs from the TbpA/Hsf-overproducing strain had significantly higher bactericidal antibody titers against H44/76 and Cu385 than mice immunized with single-overexpressed-OMP vaccines ($P \leq 0.036$) or control vaccine ($P \leq 0.0003$). When measured with strain NZ124/98, sera from mice immunized with TbpA/Hsf OMVs had significantly higher bactericidal antibody titers than sera from mice immunized with the control vaccine ($P = 0.0007$). This immunization experiment was repeated twice with similar results (data not shown).

In order to confirm the additive activity of anti-TbpA and anti-Hsf antibodies in the induction of complement-mediated killing, serum-mixing experiments were performed. For this purpose, 20 sera from mice immunized with OMVs from either TbpA- or Hsf-overproducing strains were pooled. The pools were tested alone or mixed in serum bactericidal assays against strains H44/76 and Cu385. A pool of 20 sera from mice immunized with TbpA/Hsf OMVs was used as a control. The bactericidal antibody titers measured against H44/76 and Cu385 are shown in Fig. 3A and B. The sera from mice immunized with OMVs from the strain overproducing only one minor OMP had lower bactericidal titers than the sera from mice immunized with OMVs from the strain overproducing both TbpA and Hsf and than the mixed sera. The bactericidal titers of the mixed sera and TbpA/Hsf sera were similar.

Combination of minor OMPs has synergistic effects on complement-mediated bactericidal titers. A second combination experiment was performed with OMVs from NspA- and Omp85-overproducing strains. First, pools of 20 sera from mice immunized with NspA OMVs or Omp85 OMVs were analyzed for the ability to mediate, alone or in combination, bactericidal activity against strain M97-250687 (Fig. 3C). Pooled sera from mice immunized with OMVs from strains overproducing NspA or Omp85 had low or undetectable bactericidal antibodies (titers, ≤74), but the mixture of pools displayed clear bactericidal activity against strain M97-250687 (titer, 239). Mice immunized with OMVs from the TbpA/Hsf-overproducing strain also had significant bactericidal activity against strain M97-250687 (titer determined with pooled sera, 412). To evaluate the additive effect of bactericidal antibodies directed against different minor OMPs, pooled sera from mice immunized with NspA, Omp85, and TbpA/Hsf OMVs were combined. The bactericidal activity was enhanced when the three serum pools were mixed (titer, 1,920) compared to the activity obtained with the combination of NspA plus Omp85 or the TbpA/Hsf serum pool alone (titers, 239 and 412, respectively). As observed with the other serum combinations described above, the bactericidal titer obtained with a mixture of the three sera was higher than the sum of the bactericidal titers obtained with the individual pooled sera. This indicates that there is a synergistic effect of antibodies directed against different minor OMPs, which is observed with two or more minor OMPs depending on the strain used in the bactericidal assays.

DISCUSSION

In children less than 2 years old, OMV vaccines prepared from wild-type MenB strains are able to elicit a bactericidal antibody response against homologous PorA strains but not against heterologous PorA strains (41). We developed a mouse model that mimics bactericidal antibody responses induced in infants by wild-type OMV vaccines. To avoid a PorA immunodominant response, we genetically modified strain H44/76 to knock out the *porA* gene. In this strain, the *cps* gene complex was also deleted, resulting in the absence of capsular polysaccharide and the α-chain of LOS. In our mouse model, a vaccine containing OMVs produced from this strain induced a weak or undetectable bactericidal antibody response against parental wild-type strain H44/76. The genetically modified strain was used to evaluate the impact of overproduction of minor and well-conserved OMPs on the bactericidal antibody response elicited by OMVs. We selected four minor, well-conserved OMPs with potential as vaccine antigens, which were overproduced either by promoter replacement or by gene delivery. For

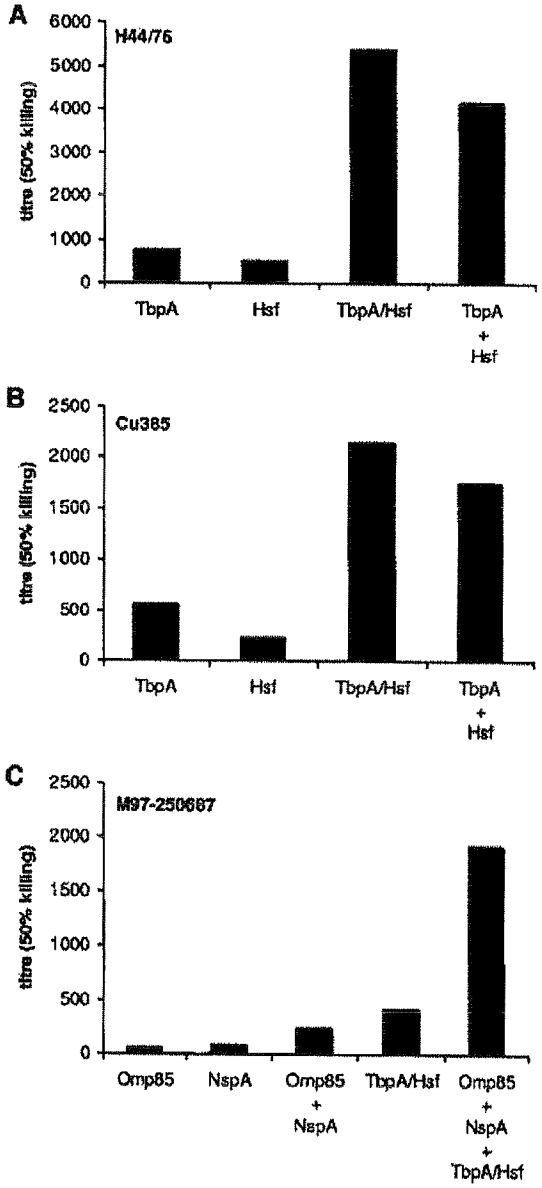


FIG. 3. Bactericidal activity against *N. meningitidis* strains of serum antibodies from mice immunized with OMV vaccines. Sera from 20 mice (see Table 4) were pooled, and two to four pools were combined. Bactericidal assays with (A) H44/76 and (B) Cu385 were performed using pooled sera from mice immunized with OMVs from either TbpA-, Hsf-, or Tbp/Hsf-overproducing strains or with a 1:1 (vol/vol) mixture of serum pools from mice immunized with OMVs from TbpA- and Hsf-overproducing strains. Bactericidal activity is expressed as the reciprocal antibody titer. (C) Bactericidal assays with strain M97-250687 were performed using pooled sera from mice immunized with OMVs from either Omp85-, NspA-, or TbpA/Hsf-overproducing strains or a combination thereof. The data are the means of three different mixing experiments performed using the same serum samples and are expressed as the reciprocal bactericidal titers.

both overexpression strategies the strong *porA* promoter was used.

We demonstrated that gene delivery or promoter replacement results in overproduction of minor OMPs in OMV preparations and that overexpression is needed to elicit the production of specific antibodies in nonprimed mice immunized with OMVs from a *porA* knockout strain. It is noteworthy that induction of antibodies against well-conserved minor OMPs, such as NspA, could also be achieved by sequential immunizations with different wild-type OMVs produced from PorA heterologous strains (25).

Using our mouse model, we observed that enhancement of the antibody response against one minor OMP may not be sufficient to mediate bactericidal activity against MenB strains even if very high specific antibody titers are induced (e.g., an ELISA titer of 51,200 for Hsf). Efficient complement-mediated killing was observed only when at least two minor OMPs were targeted by bactericidal antibodies. This was observed with anti-TbpA and anti-Hsf antibodies mediating bactericidal activity against heterologous MenB strains (Cu385, NZ124, and M97-250687) and also with anti-NspA and anti-Omp85 antibodies (tested only against strain M97-250687). Moreover, increasing the number of targeted minor antigens on the surface of the bacteria leads to further enhancement of bactericidal antibody titers, which are clearly higher than the simple sum of bactericidal titers obtained when only two minor OMPs are targeted. Our hypothesis is that a minimal density of bacterial surface proteins occupied by bactericidal antibodies must be reached to allow activation of the complement cascade via the classical pathway (Fig. 4). We suggest that activation of complement requires at least two adjacent antibodies, which do not necessarily have to be directed against the same antigens. However, the corresponding antigens should be close enough to each other on the bacterial surface to allow binding of a C1q molecule to at least two Fc domains, which is the first step in the initiation of the classical complement cascade (6). This situation is probably more characteristic of coccal bacteria. For example, bacillary and coccobacillary bacteria have polar localization of autotransporter proteins, but in coccal bacteria, such as *N. meningitidis*, expression of these proteins is observed at different loci (19). Consequently, when only one minor meningococcal OMP is targeted, the bactericidal antibodies are scattered over the surface of the bacteria and would not be able to fix the C1q factor, which is the first step of the classical pathway activation of the complement cascade leading to the formation of the membrane attack complex. To increase the density of bactericidal antibodies on the surface of the bacteria, our strategy was to target several minor, well-conserved OMPs simultaneously. This strategy could also be applied to other gram-negative bacterial species that naturally do not produce blebs by producing mutations in the *tol-pal* genes that result in the formation of blebs (2, 9).

The overproduction of several well-conserved minor OMPs induced a cross-reactive bactericidal antibody response, which has two advantages for a MenB vaccine with broad coverage. First, it allows interference with several stages of the meningococcal infection process, such as iron uptake (via TbpA), adhesion (via Hsf and possibly NspA), and vital function (via Omp85). Second, it reduces the risk of emergence of vaccine escape mutants.

In conclusion, we have demonstrated that it is possible to induce a protective bactericidal antibody response against MenB strains by immunization with OMVs from strains lacking the major, variable PorA OMP and overproducing selected

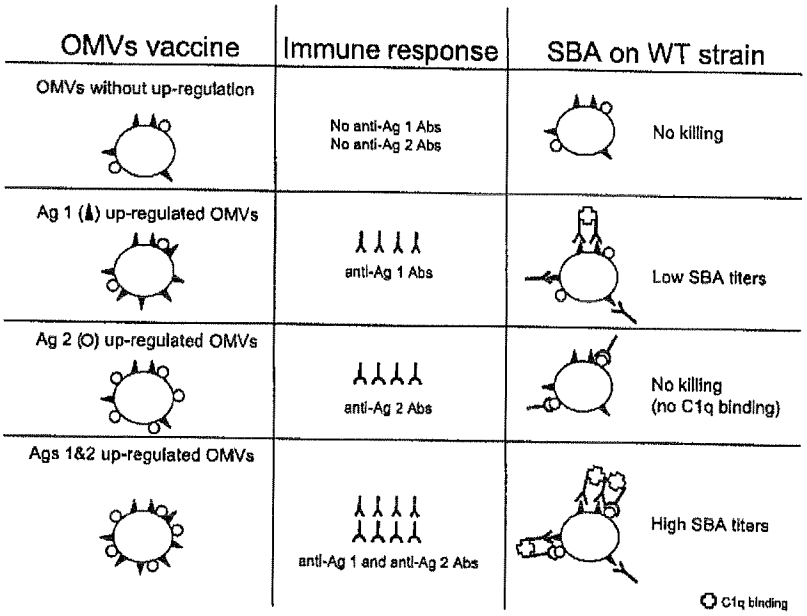


FIG. 4. Schematic representation of the impact of the antigen density on the bactericidal activity of antibodies on the surface of coccal MenB strains. A defined density is required to induce significant bactericidal antibody killing mediated by complement. This threshold density is reached when at least two minor OMPs (Ag 1 and Ag 2) are targeted by antibodies. For an efficacious OMV vaccine, at least two minor OMPs must be overproduced, resulting in vaccine-induced bactericidal antibody killing. Ag, antigen; Abs, antibodies; SBA, serum bactericidal activity. The first step of the classical pathway for activation of the complement cascade is binding of the C1q factor to antibodies, leading to the formation of the membrane attack complex.

well-conserved minor OMPs. Our results for a limited number of heterologous strains also suggest that the use of well-conserved minor OMPs results in a cross-protective response. This approach may be the key to development of a fully protective vaccine for meningococcal disease and may also be a strategy that is generally applicable to other host-adapted bacterial pathogens in which phase and antigenic variation of major OMPs has stifled vaccine development.

ACKNOWLEDGMENTS

M.P.B. is supported by The Netherlands Council for Chemical Sciences (CW) with financial aid from The Netherlands Technology Foundation (STW). Work in M.P.J.'s lab is supported by program grant 284214 from the National Health and Medical Research Council of Australia. This work was supported in part by GlaxoSmithKline Biologicals, Rixensart, Belgium.

We thank U. Krause for editorial assistance.

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Editor: J. N. Weiser